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To Grow or Defend? Effects of Competition and Herbivory on Common Milkweed, Asclepias syriaca

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ABSTRACT

Plants experience trade-offs between growth, maintenance, reproduction, and defense (Herms & Mattson 1992, Mauricio 1998, Strauss et al. 2002). Defenses can be mechanical or chemical, and include production of trichomes, toxic cardiac glycosides (cardenolides), and latex. We chose common milkweed, *Asclepias syriaca* as our study system because it exhibits inducible defensive responses to herbivory (Wang et al. 2014). To gain a better understanding of the drivers of escalation in defenses in common milkweed, *Asclepias syriaca,* we tested three hypotheses: (1) increased plant density results in competition which lowers plant defenses to herbivory (2) natural and simulated herbivory should induce the same defensive responses in milkweed *as long as the simulated herbivory is done in the same relative amount and to the same plant tissue as natural herbivory* and (3) increased herbivory intensity should induce greater defensive responses in common milkweed. Plant size data was collected for all plants, and defenses were sampled three times within a 72-hour timeframe. Our density treatments showed that competition has significant effects on all measures of size: plants in the low-density treatments were consistently larger than those in the high-density treatment. Competition effects on leaf traits, indicators of plant tissue quality, were variable between our studies. Competition did not have effects on plant defenses. Herbivory Type had an effect on some, but not all leaf traits, and had no effect on latex exudation. Herbivory Intensity was found to have a positive relationship with leaf traits and latex exudation. Further investigation needs to be done on the effects of these treatments on cardenolides, as these were highly variable and there were no relationships between any of our treatments and cardenolide concentration. In the

Herbivory Type Study, this would support the conclusion that simulated herbivory can sufficiently mimic natural herbivory by monarch caterpillars if applied properly.

INTRODUCTION

Plants experience trade-offs between growth, maintenance, reproduction, and defense (Herms & Mattson 1992, Mauricio 1998, Strauss et al. 2002). Production of defenses, then, may result in reduced interspecific and intraspecific competitive ability in terms of fitness traits such as growth or reproduction (Strauss et al. 2002). This tradeoff is supported by the resource availability hypothesis in which defense investment increases as potential growth rate of the plant decreases (Coley et al. 1985). Competition among plants imposes stress on individuals as a result of reduced resource availability, and they consequentially are forced to do one of two things: growth or defend (Siemens et al. 2002, Herms & Mattson 1992). Although ecological theory posits that intraspecific competition should affect the tradeoff between growth, reproduction, and defense, there is a scarcity of studies that examine the effects of density (i.e., intraspecific competition) on the expression of defensive traits.

Plant defense traits can include forms of mechanical protection and/or chemical protection. Chemical defenses in the forms of secondary metabolites are the primary reason why some plants are able to escape herbivore damage (Herms & Mattson 1992, Bernays & Chapman 1987). Chemical defenses can be either inducible (i.e., they change after a stimulus) or constitutive (i.e., present at all times). Plants with an inducible defense may have an advantage over those with constitutive defenses because plants with inducible defenses can re-direct resources to costly defenses only when necessary, freeing resources for growth and reproduction when the plant is not under stress (Herms & Mattson 1992). Plants with low constitutive levels of defenses (and/or with the ability to increase those defenses if need be) would be expected to

have greater growth and reproduction than those with high constitutive levels of defense (Holeski et al. 2013, Stamp 2003).

Common milkweed, *Asclepias syriaca* is an ideal study system for understanding how these trade-offs play out for several reasons. First, defensive characteristics can be either constitutive or induced in common milkweed. Their resistance traits include trichomes, latex exudation, and toxic secondary compounds called cardenolides (Agrawal et al. 2008, Agrawal et al. 2009b, and Dalin et al. 2008). These are representative of both mechanical as well as chemical protection. Trichomes are hair-like structures that limit herbivory (Agrawal et al. 2009a). Latex is a white substance from which milkweed derives its name, and has negative effects on herbivore chewing due to its sticky nature as well as the cardenolides it carries (Agrawal et al. 2008). The cardenolides belong to a family of secondary compounds called cardiac glycosides (Nelson 1993, Wiegrebe & Wichtl 1993). Although cardenolides are known to occur in various parts of the adult plant, they have been most intensively studied in the leaves (Agrawal et al. 2012a).

Interspecific variation in plant defenses, specifically cardenolide content has been shown to correspond to the amounts of sequestered cardenolides in adult monarch butterflies (Brower et al. 1972). Additionally, we know that there exists variability of plant defenses within the genus *Asclepias* (Agrawal 2004a, Zehnder & Hunter 2007). Many studies have shown that specialist herbivores like the monarch are negatively affected by plant defenses (Zalucki et al. 1990, Zalucki & Brower 1992, Zalucki et al. 2001). Additionally, one study found negative genetic correlations between cardenolide production and plant growth (Züst et al. 2015). There is a lack of

understanding of how intraspecific competition drives production of defenses in common milkweed, which is a prudent direction of study considering our understanding of how the defenses (particularly cardenolides) impact herbivores.

Finally, common milkweed is an important study system due to its relevance in the life cycle of the monarch butterfly, *Danaus plexippus*. The monarch is a migratory species that travels from eastern breeding grounds in the United States and Canada to overwintering sites in Mexico. According to a recent study by Flockhart et al. 2014, habitat loss in the summer breeding grounds in the U.S. are a driver of the population decline observed over the past decade. As a major food source for larval monarchs during the summer breeding season in North America, common milkweed is especially significant (Lynch 1987). Prior to their decline, monarchs were extensively studied because they are aposematic as a result of sequestration of toxic cardiac glycosides in the milkweed they feed on as larvae (Malcolm & Brower 1989). The milkweeds, genus *Asclepias*, are primary host plants for monarch breeding (Agrawal & Malcolm 2002). There are 27 species of milkweed in North America that monarchs feed on as larvae, however they are most efficient at sequestering the cardiac glycosides from common milkweed *A. syriaca* (Malcolm & Brower 1989). Common milkweed exists along roadsides and in agricultural field edges (Hartzler & Buhler 2000). Milkweeds have suffered drastic declines in the U.S. as a result of increased planting of glyphosate-tolerant, genetically modified corn and soybeans (Pleasants & Oberhauser 2013). Recent literature surrounding monarchs has focused on demonstrating how monarch populations have been linked to habitat loss in its breeding grounds in the United States (Flockhart et al. 2014).

A deeper understanding of the relationship between monarchs and their host plants (milkweed) is necessary given the dwindling populations of both partners of this relationship. The relationship between monarchs and milkweeds is biologically interesting and has been compared to an arms race (Agrawal 2005). Monarchs are able to tolerate the toxic secondary compounds (cardenolides) preset in milkweed, but high concentrations are still deleterious to monarch growth (Dalla et al. 2013). An understanding of the effects of competition on defenses as well as growth for common milkweed would thereby contribute to general understanding of the drivers of monarch success and preference for this species.

We had three objectives for two studies. The first objective, tested in both studies, was to explore the relationship between resource availability and defensive responses of milkweed. Our hypothesis was that increased plant density results in competition, which will lead to lower defensive responses to herbivore damage by milkweed. The second objective is a validation of methods used for simulated herbivory treatments in previous studies (Malcolm & Zalucki 1996, Zalucki & Brower 1992). Methods used to study inducible responses are highly variable. Many studies have used insect herbivores to induce defenses (Agrawal 2004b, Rasmann et al. 2009, Zhender & Hunter 2007). Some simulate herbivory by manually severing the leaf partially (Malcolm & Zalucki 1996, Zalucki & Brower 1992). Those papers which used simulated herbivory (Malcolm & Zalucki 1996, Zalucki & Brower 1992) have made the assumption that simulated herbivory causes the same responses in milkweed as natural herbivory by insects. Our hypothesis was that natural and simulated herbivory should induce the same defensive responses in milkweed as long as the simulated herbivory is done in

the same relative intensity, and to the same plant tissue, as the natural herbivory it seeks to mimic. This was tested in the study performed at the College of William & Mary, and will be referred to as the Herbivory Type Study. The third objective was to explore the effects of herbivory intensity on defensive responses of milkweed. This was tested in the study performed at University of Maryland- College Park, and will hereafter be referred to as the Herbivory Intensity Study.

METHODS

Species Description

Milkweed

Common milkweed, *A. syriaca,* is a perennial plant native to North America. It prefers edge habitats in eastern portions of both the United States and Canada. Milkweed can reproduce both sexually and clonally; sexual reproduction occurs by hermaphroditic flowers and clonal reproduction is through adventitious root buds (Agrawal 2005). A single pollination event during sexual reproduction in milkweed leads to the production of a seedpod on the plant. We have seen a range of 72 to 126 seeds in a pod, all of which are full siblings.

The cardenolides are a group of toxic steroids carried in the leaf cells and latex, a sticky white substance that limits herbivore damage (Agrawal et al. 2012b). Cardenolides are comprised of a 23-carbon structure with a steroid backbone, a five membered lactone ring attached to C17, and a sugar attached to C3 (Agrawal et al. 2012b). Collectively, these three resistance traits have led to the establishment of a

small guild of specialist herbivores that have ways to ameliorate or tolerate milkweeds' defenses (Agrawal 2005). The monarch butterfly, *D. plexippus,* is one such herbivore.

Monarch Butterfly

As a specialist herbivore to *Asclepias,* the monarch is able to tolerate its defensive traits. This tolerance is limited, however, and monarch larvae feeding on milkweed have evolved behaviors to reduce ingestion of the trichomes, latex, and toxic cardenolides (Zalucki 2001). To avoid consuming the hairy trichomes, monarch caterpillars shave away trichomes before beginning to actually eat the leaf (Agrawal 2012b). The caterpillars methods of avoidance of latex and cardenolides are variable with instar. First instar caterpillars have the poorest survival because of latex and cardenolides (Zalucki and Brower 1992). The avoidance behavior of the first-instar caterpillars is known as trenching, during which the caterpillar chews notches in the shape of a circle in a leaf, and then eats the center which no longer has latex flowing to it (Agrawal 2012b). Larger instar caterpillars forgo this behavior in favor of severing leaf petioles followed by feeding on the tissue distal to the severed area (Zalucki 2001).

Study 1. Herbivory Type

Seeds of common milkweed, *Asclepias syriaca,* were taken from field sites collected in the summer of 2013. Three seedpods were used from different site locations: one from Yorktown National Battlefield in Virginia and the other two from the same plant at Presquile National Wildlife Refuge, Virginia. Seeds were counted and

cold stratified in moist sand at 4 degrees Celsius for a ninety-day period over the winter of 2013-2014. The seeds were then planted in plug trays and grown for six weeks under grow lights in the William & Mary greenhouse. Once the plants had all reached approximately ten centimeters in height, they were transplanted into 15-gallon tubs. All tubs contained a racemic mixture of Sunshine LCL mix and Metro-Mix PX1 (Sun Gro Horticulture, Vancouver, BC, Canada). All tubs were treated with slow release Osmocote Fertilizer mixed into the uppermost portion of the soil. 92 grams of Osmocote Plus was used. Osmocote content for nitrogen, phosphorous, and potassium are as follows: Total N 15%, Phosphate (P_2O_5) 9%, Soluble potash (K_2O) 12%.

All tubs were all kept in the William & Mary greenhouse. During the summer of 2014, cages made out of a PVC frame wrapped by mesh netting individually protected tubs. The cages did not have a top to allow sun to reach the plants. The cages were used to increase the efficacy of the herbivory treatments by preventing movement of herbivores between tubs. The netting also served to protect plants from unwanted herbivory by other insects. If any such herbivores did appear (most commonly seen were Aphids, *Aphis nerii*) they were brushed off using a paintbrush. Plants were then treated with an organic soap wash. This soap wash was removed by hand washing of all leaves before the herbivory treatment in July.

Competition & Herbivory Treatments

Milkweed was grown in low and high-density treatments of three plants per tub and twelve plants per tub, respectively. There were nine of each density type for a total

of eighteen tubs, which were further sub-divided into herbivory treatments: no herbivory (control), simulated herbivory (manual herbivory done by ripping of apical leaves) and natural herbivory (performed by Monarch butterfly caterpillars). Each herbivory treatment was replicated on three randomly assigned low-density and high-density tubs. The herbivory treatments were performed in two time frames. The first herbivory treatment was natural herbivory by monarch caterpillars. The simulated herbivory treatment was performed a week after natural herbivory so that data could be collected on the amount of damage inflicted by the caterpillars in the first 24-hour period. The caterpillars were allowed to feed on the plants (one caterpillar per plant) for up to 72 hours, and plants were sampled at the 24, 48, and 72 hour marks. The control plants were sampled at the same time as the natural herbivory plants. Damage (percent leaf area lost to herbivory) was recorded for all natural herbivory treated plants and averaged to create an estimate to be "mimicked" in the simulated herbivory treatment. The average damage inflicted by monarch caterpillars in the first 24 hours was twenty percent leaf area. A week after the natural herbivory treatment, simulated herbivory was performed on the pre-selected tubs by ripping twenty percent of the most apical fully extended leaves.

Study 2. Herbivory Intensity

Seeds of common milkweed, *Asclepias syriaca,* were received from the seed program at the North Carolina Botanical Program at University of North Carolina Chapel

Hill in 2013. The seeds were cold stratified in moist sand at 4 degrees Celsius for a two-month period. Seeds were planted directly in treatment tubs, and any that did not germinate were compensated for using transplanted plants. All tubs contained a racemic mixture of Sunshine LCL mix and Metro-Mix PX1 (Sun Gro Horticulture, Vancouver, BC, Canada). In 2014, the plants were treated with Oscmocote, a slow release fertilizer.

All tubs were kept at the University of Maryland greenhouse. During the growing season they were housed in the greenhouse, overwinter they were kept outside. During the summer of 2014 the tubs had PVC frames with mesh netting to protect plants, however these were taken down by July owing to plant weakness. This, we believe, was because the shading provided by the netting was deleterious to plant growth. On June 16, 2014 the plants were all cut back due to an infestation of thrips. For the purposes of this study, the age of the plants begins on June 16, 2014 because of this cutting.

Competition and Herbivory Treatments

Milkweed was grown in incremental densities. These were 1 plant per tub, 3 plants per tub, 6 plants per tub, and 12 plants per tub. Average density in natural populations of milkweed are around 3.15 plants per square meter (H.J. Dalgleish, unpublished data). The densities in the Herbivory Intensity Study would correspond to a range of densities between 1 and 50 plants per square meter. Each density was replicated four times. Herbivory intensity treatments were randomly assigned for each pot. The levels of herbivory were none, low (10% of leaf mass removed from the top two

nodes), medium (50% of leaf mass removed from the top three nodes), and high (75% of leaf mass removed from the top five nodes). On the day herbivory treatments were performed, thrichome samples were also collected from all pots except for those assigned to no herbivory. For each plant, the opposite leaf on the top node from which trichome samples were collected was left untouched so that latex could later be collected from that leaf. For each pot, the time herbivory was performed was recorded, then the leaves were either hole punched or torn by hand until the right amount of leaf area had been removed for each treatment.

Data Collection: Both Studies

Demographic measures were taken for all plants in both studies: plant height to the apical meristem, basal stem diameter, number of leaves, longest leaf length, and longest leaf width. Samples for leaf trichomes, latex, and cardenolides were all taken during each sampling period. Leaf trichome samples were taken by using a hole-punch from the most apical fully extended leaf. These samples were stored in a freezer at -80 degrees Celsius. Latex exudation was measured onto a $1cm^2$ piece of pre-weighed filter paper, after cutting the first 5 mm off the tip of the youngest fully extended and intact leaf (Rasmann et. al 2009). Cardenolide samples were collected by removal of the youngest fully extended leaves. These were kept on ice in a cooler during sampling and then immediately stored in a freezer at -80 degrees Celsius. Once they were frozen the plant tissue samples were lyophilized and stored until extraction. Samples consisting of 150 mg of plant tissue were sent to University of Wisconsin, Madison for spectral analysis (described below). Remaining plant tissue was used for extraction.

Extraction

The frozen plant material was ground using an Omni Bead Ruptor 24 ball-mill (Omni International). 50 mg (+/-5 mg) of ground plant material was spiked with 20 µg of digitoxin (Sigma) as internal standard, which was extracted with 1.9 mL of 95% ethanol in a sonicating water bath for 20 min. Ethanol was evaporated overnight in a fume hood, and residue was dissolved in 0.5 mL methanol (Rasmann et al. 2009).

High Performance Liquid Chromatography

Extracted samples were analyzed using a Shimadzu HPLC (High Performance Liquid Chromatographer) with an LC system and diode array detector. Samples were eluted at a constant flow of 0.7 mL/min with a buffer of 0.25% phosphoric acid in water and acetonitrile in the following gradients: 0-5 min 20% acetonitrile, 20 min 70% acetonitrile, 20-25 min 70% acetonitrile, 30 min 95% acetonitrile, 30-35 min 95% acetonitrile. UV absorbance spectra were recorded from 200 to 400 nm by diode array detector. Symmetric peaks with absorption maxima between 218 and 225 were recorded as cardenolides and quantified at 218 nm (Agrawal 2014, Wiegrebe & Wichtl 1992, Rasmann et al. 2009, Rasmann et al. 2011).

Spectral Analysis

Spectral analysis was performed at University of Wisconsin-Madison using the methods from Couture et al. 2013. Concentrations of cardenolides were measured

using a microplate reader to adapt methods from Brower et al. 1972 and Nelson 1993. This approach uses absorbance of a chromophore created from the reaction between the butenolide part of cardenolides and 2, 2',4,4'-tetranitrodiphenyl, or TNDP (Couture et al. 2013). TNDP extracted and reacted samples are compared with blank samples and extracted samples without TNDP and the difference in absorption is measured using an external curve (Couture et al. 2013). Spectral analysis provided measures of leaf traits including lignin, fiber, cellulose, total carbon, and nitrogen content.

RESULTS

Herbivory Type Study

Effects of Intraspecific Competition on Plant Size in Common Milkweed

There was strong evidence of intraspecific competition due to density in all four of our measures **(Figure 1)**.

The mean basal stem diameter (BSD) was larger in low density tubs than in high density tubs: Low = 5.90 ± 1.59 High = 3.74 ± 1.55, ($P < 0.001$, $F_{1,54} = 25.25$) The same was true for Height (cm): Low = 59.81± 18.17 and High = 44.31 ± 19.96 ($P = 0.005$, $F_{1,54} = 8.64$) There was also a greater number of leaves (per plant) in low density tubs than high: Low = 25.41 ± 5.02 and High = 15.76 ± 6.90 ($P = 6.1e^{-7}$, $F_{1,54} = 31.96$) Finally, Leaf Area (cm^2) was significantly greater in the lower density than high density: Low = 44.47 ± 13.32 and High = 27.81 ± 14.98 ($P < 0.001$, $F_{1,54} = 17.97$).

Effects of Intraspecific Competition on Leaf Traits on Common Milkweed

Intraspecific competition did not have an effect on all measures of plant leaf traits (lignin, total carbon, nitrogen, leaf mass per area, cellulose, and fiber; **Figure 2**)**.** Of these, there was only a significant difference in total carbon: Low = 44.20 ± 0.72 High = 45.01 ± 1.06 ($P = 0.02$, $F_{1,30} = 5.78$). This shows the reverse trend from what was seen in the plant growth, with a greater amount of biomass in high-density treatments.

Effects of Intraspecific Competition on Plant Latex Exudation

There was no effect of density treatment on latex exudation (P = 0.80, $F_{1,54}$= 0.07). Latex was highly variable, but that variation was not explained by differences in plant size.

Effects of Herbivory Type on Plant Leaf Traits

Simulated herbivory resulted in higher values for several leaf traits, including lignin, fiber, and total carbon **(Figure 5)**. Plants treated with simulated herbivory had higher lignin than caterpillar or control (P = 0.002, $F_{2,30}$ = 7.323). The same was true for fiber (P = 0.001, $F_{2,30}$ = 8.313) as well as total carbon (P = 0.02, $F_{2,30}$ = 4.46). There was no relationship between herbivory type and cellulose (P = 0.13, $F_{2,30}$ = 2.20) or total nitrogen (P = 0.60, $F_{2,30}$ = 0.54).

Effects of Herbivory Type on Plant Latex Exudation

There was no relationship found between herbivory type and latex exudation (P = 0.298, $F_{2,54}$ = 1.24).

Effects of Herbivory Type, Plant Density, and Time on Cardenolide Concentration

Herbivory Type did not have any effect on cardenolide concentration in *A. syriaca* in our study (P = 0.847, $F_{2,54}$ = 0.166). Additionally, we did not see a significant effect of plant density (P = 0.179, $F_{2,54}$ = 1.852) nor time (P = 0.341, $F_{2,54}$ = 1.15). These results are shown in **Figures 9 and 10.**

Herbivory Intensity Study

Effects of Intraspecific Competition on Plant Size in Common Milkweed

The larger number of density treatments in this study support the results from the study at William & Mary **(Figure 3).** Of the four measures of plant size (basal stem diameter, height, number of leaves, and leaf area) there was a significant relationship between density and BSD, number of leaves, and leaf area. Height was not significantly affected by density treatment (β = 0.23, SE = 0.92, P = 0.80, $F_{1,188}$ = 0.06). Basal stem diameter was lower in high density tubs than in low density tubs (β = -0.24, SE = 0.12, P = 0.05, $F_{1,188}$ = 4.70) Plants had more leaves in low density tubs than high density tubs (β = -1.17, SE = 0.56, P = 0.04, $F_{1,188}$ = 4.32) Finally, leaf area was smaller in highest density treatments as compared to lowest density treatments (β = -3.92, SE = 1.43, P = 0.006, $F_{1,188}$ = 7.50).

Effects of Intraspecific Competition on Leaf Traits in Common Milkweed

Intraspecific competition had a very different relationship on several measures of plant leaf traits for the Herbivory Intensity Study compared to the Herbivory Type Study. The effect of plant density on total nitrogen was the most significant (β = -0.08, SE = 0.02, P < 0.001, $F_{1,84}$ = 17.93, **Figure 4**). Total carbon was also significant (β = -0.09, SE = 0.03, P = 0.01, $F_{1,84}$ = 6.31). Lignin was significant at the 0.1 level (Lignin: β = -0.22, SE = 0.13, P = 0.09, $F_{1,84}$ = 2.99). Competition did not have significant effects on cellulose or fiber.

Effects of Intraspecific Competition on Plant Latex Exudation

As with the Herbivory Type Study, there was no effect of density treatment on latex exudation (β < 0.001, SE = 0.0002, P = 0.74, $F_{1,188}$ = 0.11). Latex was highly

variable and no correlation was found between latex exudation and measures of plant growth.

Effects of Herbivory Intensity on Plant Leaf Traits

Lignin, fiber, and total carbon were affected by the herbivory intensity. In the Herbivory Intensity Study, Lignin was lowest in the plants with the lowest herbivory amount (*β = 1.57,* SE = 0.44, *P < 0.001, $F_{1,84}$ = 12.48,* **Figure 6**). The same pattern held true for fiber (β = 1.40, SE = 0.54, *P* = 0.01*, $F_{1,84}$* = 6.67). Total carbon also increased with herbivory intensity (β = 0.26, SE = 0.12, *P* = 0.04*, $F_{1,84}$* = 4.48). Finally, cellulose and total nitrogen did not have significant differences based on herbivory amount.

Effects of Herbivory Intensity on Plant Latex Exudation

When herbivory amount was treated as a continuous variable in a linear model as herbivory intensity increased latex exudation increased as well (β = 0.001, SE = 0.0002, *P* < 0.001, $F_{1,188}$ = 20.53 **Figure 7**).

Effects of Herbivory Intensity and Plant Density on Cardenolide Concentration

The Herbivory Intensity Study had a limited number of samples that were used for cardenolide analysis. At this point, there was only one sample from the 72 hour time mark from the Herbivory Intensity Study. Because of this, we looked at the combined effects of only herbivory intensity and plant density on cardenolide concentration. There was no interaction between intensity and density on cardenolide concentrations (*P* = 0.574, $F_{7,22}$ = 0.830). Herbivory intensity did not have a significant effect on cardenolide

concentrations in *A. syriaca* ($P = 0.595$, $F_{1,38} = 0.64$), however as can be seen with other results from this part of the experiment (**Figure 8**) there is an increasing trend in cardenolide concentration as herbivory intensity increases from no herbivory to high herbivory. There was no difference in mean cardenolide concentration based on plant density treatment ($P = 0.759$, $F_{1,38} = 0.467$). Additional cardenolide data is forthcoming which will allow us to examine any effects of time on cardenolide concentration in the Herbivory Intensity Study.

Results of Time: Both Studies

We took samples at three time points: 24, 48, and 72 hours after herbivory or simulated herbivory. For both the Herbivory Type and the Herbivory Intensity studies, time (sampling period) had no effect on latex exudation alone or in conjunction with plant density or herbivory treatment (Herbivory Intensity: $P = 0.12$, $F_{2,33} = 2.11$, Herbivory Type: $P = 0.35$, $F_{2,53} = 1.08$). Additionally, there was no effect of time on most measures of leaf traits. The two exceptions (out of all measures of leaf traits for both studies) were both in the Herbivory Type Study. These were lignin and cellulose ($P = 0.06$, $F_{1,30} = 2.28$) and ($P = 0.01$, $F_{1,30} = 2.80$) respectively.

DISCUSSION

We used *Asclepias syriaca* as a model system for our experiments. Our studies had three major objectives: to test for a relationship between competition and resource allocation to defensive responses, to compare the defensive responses to natural

versus simulated herbivory, and to test for a relationship between herbivory intensity and defensive responses.

Objective 1: effects of competition on common milkweed

In both the Herbivory Type and Herbivory Intensity Study we observed a strong effect of intraspecific competition on all measures of plant size. In both studies as plant density increased, plant size decreased. Leaf traits increased in value for the plants experiencing more intense competition in the Herbivory Type Study. In the Herbivory Intensity Study, all leaf traits showed a decreasing trend as competition increased and several of these has significant slopes. We expected all leaf traits to decrease as plant density increased. There are several caveats to note for the differences in leaf traits between the two studies. First, the plants from the Herbivory Intensity Study are two years old, a full year older than the plants in the Herbivory Type Study. They experienced competition over a longer period of time than the plants in the Herbivory Type Study, and the effects on leaf traits might be long-term effects of competition.

Competition did not impact latex exudation in either study. Soil moisture and fertilization have been documented to affect latex production in *Asclepias* species (Agrawal & Kono 2009). We applied fertilizer in both studies and watered all treatments equally. Since we did not see a difference in latex and latex is at least partly dependent on soil moisture (Agrawal & Kono 2009), we can attribute our results to our plants having sufficient water even in the high density tubs. Collection of analysis of latex exudation from plants experiencing different levels of competition should be replicated

to see if these results are replicable, and incorporation of a water deprivation treatment would help validate our assessment.

In the Herbivory Type Study, mean foliar cardenolide concentrations showed a trend that indicated that there might be lower cardenolides in plants that are heavily competing for resources. We were limited in sample size for the Herbivory Intensity Study cardenolide analysis, so while we did not see an effect of competition on cardenolide concentration, we need more data to form any conclusions from this study. We expected one of two things to happen: (1) plants experiencing competition would be smaller and allocate more resources to defense or (2) plants experiencing competition would allocate more resources towards growth in order to compete for light and would allocate fewer resources to defense (Züst et al. 2015). Competition did not have any effect on cardenolides, and in both studies there was no correlation between any measure of plant size and cardenolides. The trend observed in the Herbivory Type Study is compelling for further research and greater data collection, since we had a limited number of samples to analyze using HPLC.

Objective 2: Effects of herbivory type on common milkweed

In the Herbivory Type study, plants treated with simulated herbivory had significantly higher values for Lignin, Fiber, and total Carbon. That we are aware of, there is no explanation for why might happen, and might be due to small sample size. The Herbivory Type Study also has limited sample size due to greenhouse space. Additionally, allocation of biomass has been shown to be variable across species, and can be affected by conditions such as shade (Dias-Fihlo 200). Leaf traits, especially

total carbon and nitrogen, are indicators of plant biomass allocation (Mattson 1980). The effects of competition on the size of our plants, particularly the high variation in height and basal stem diameter in high competition tubs, indicates that our plants were likely affected by the shading from the mesh cages we used. Exploring the effects of shade on plant biomass allocation in common milkweed would be an interesting future direction. Since it has not been seen in the literature but has been shown to impact allocation of biomass in other species, this might confound our already limited sample size. One of the primary differences between using simulated and natural herbivory is that simulated herbivory is only performed once. Mattson 1980 showed that increased herbivory causes some plants to allocate more towards biomass in the form of replacement tissue, so we might have expected plants being continuously damaged by herbivores to allocate more resources towards biomass. The results of this portion of the study might also have been impacted by our short timeframe. This idea requires further testing on the effects of simulated versus natural herbivory on leaf traits.

Herbivory Type did not elicit a significant response in milkweed in the form of latex exudation. We expected to see plants experiencing natural herbivory to have escalated latex exudation due to constant herbivore pressure (Agrawal et al. 2008, Rasmann et al. 2009).

In the Herbivory Type study, there was no effect of herbivory on cardenolide concentration. Our results indicate that the simulated herbivory elicits the same response as natural herbivory. This is an important finding that should be replicated with further testing, but is important for two reasons: (1) past studies have used simulated herbivory while operating under the assumption that it elicits the same defensive

responses by common milkweed and (2) at least one previous study (Schat & Blossey 2005) has shown that simulated herbivory does not induce the same response as natural herbivory, however this study was done in comparison to beetle damage, not caterpillar damage. Our results are not entirely surprising since we sought to mimic both type and amount of foliar tissue taken during the first 24 hours of natural herbivory. In the Herbivory Type study, we see that cardenolides are most concentrated in foliar tissue after 48 hours. We expected to see higher cardenolides at the 72 hour mark since cardenolide escalation has been shown to be most pronounced between 3 and 5 days (Agrawal et al. 2014).

Objective 3: effects of herbivory intensity on common milkweed

In the Herbivory Intensity study the plants that received the greatest intensity of herbivory (most amount of foliar tissue removed) had significantly higher Lignin and Fiber than those that received no herbivory. Plant biomass and allocation to growth have been shown to escalate with herbivory, and it has been hypothesized that this is a compensatory effect by the plant in response to tissue loss (Schierenbeck et al. 1994). The results from the Herbivory Intensity Study seem to support the hypothesis from Schierenbeck et al. 1994.

High herbivory intensity resulted in greater latex exudation. This fit our hypothesis that more damaged plants should allocate more resources to defense. In comparison to the Herbivory Type Study, the amount of tissue removed in the Herbivory Intensity Study was greater, and damage was done at only one time period. We did not see an effect of time on latex exudation in the Herbivory Type Study, but expected to

see that plants receiving natural herbivory showed increased latex exudation over time. This did not happen in the Herbivory Type Study. Differences in the age of the plants between the two studies should be taken into consideration here since we did not observe the same trends in both studies. Additionally, the plants from the Herbivory Intensity Study received the same amount of damage in their first year of growth, so we might be seeing a lag effect in defensive response.

In Herbivory Intensity Study, we saw a trend that indicated that high amounts of herbivory resulted in decreased defensive response by the plant in the form of secondary metabolites. This contradicts our findings from the latex exudation, and since cardenolides are carried in the latex seems confounding. We had a limited number of samples which we were able to quantify cardenolide concentration for in the Herbivory Intensity Study. Therefore, we cannot conclude that herbivory intensity has any effect on cardenolide concentration since it could be attributable to variation within the limited sample size. We did not find any relationship between biomass and cardenolide concentration but were very limited in sample size, but this warrants further investigation, however, since we saw that increases in herbivory intensity had a positive relationship with several measures of plant biomass allocation.

Conclusions

Competition had strong effects on all measures of plant size, however it resulted in variable responses in the form of biomass allocation (i.e. leaf traits) between the two studies. We did not observe an effect of competition on latex exudation in either study, nor in the cardenolide concentration. Herbivory Type had a relationship with biomass

allocation in our study, however this was limited in sample size and needs more data. Herbivory Type additionally had no effect on latex or cardenolide concentration. We would expect these results to be replicable (although more data is needed) and believe that by mimicking type and amount of natural herbivory manually the same defensive responses can be induced in common milkweed. Finally, Herbivory Intensity had a positive relationship with plant biomass allocation, and led to increased latex exudation in common milkweed. We did not see an effect of herbivory intensity on cardenolide concentration, but had a limited sample size so we need more data to make any conclusions.

Figures

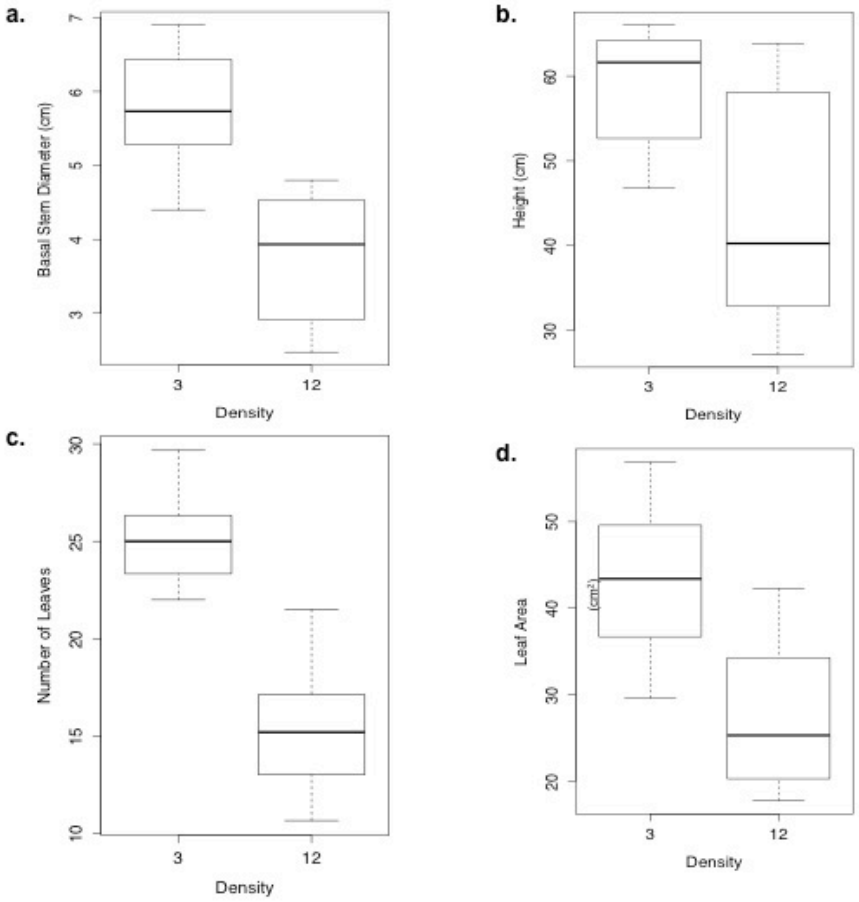


Figure 1. Intraspecific Competition Effects and Plant Size, Herbivory Type Study. **(a)** Basal Stem Diameter (cm): $P < 0.001$, $F_{1,54} = 25.25$ **(b)** Height (cm): $P = 0.005$, $F_{1,54} = 8.64$ **(c)** Number of Leaves (per plant): $P < 0.001$, $F_{1,54} = 31.96$ and **(d)** Leaf Area (cm^2): $P < 0.001$, $F_{1,54} = 17.97$

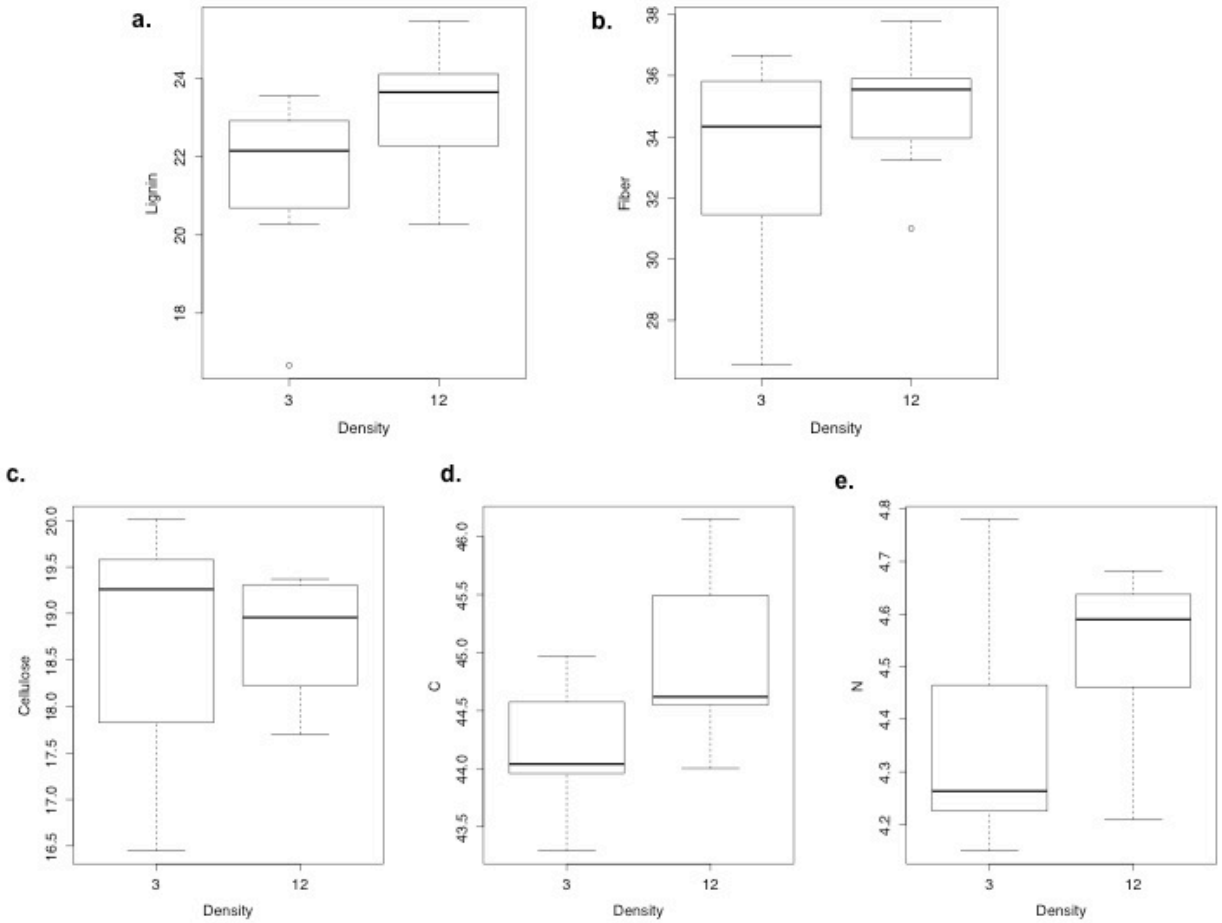


Figure 2. Intraspecific Competition Effects on Plant Leaf Traits, Herbivory Type Study. These graphs show plant density (x axis, 3 and 12 plants per tub) and the mean values for 5 measures of plant nutrients. **(a)** Lignin $P = 0.11$, $F_{1,30} = 2.72$ **(b)** Fiber: $P = 0.25$, $F_{1,30} = 1.40$ **(c)** Cellulose: $P = 0.87$, $F_{1,30} = 0.03$ **(d)** Total Carbon (C): $P = 0.02$, $F_{1,30} = 5.78$ and **(e)** Total Nitrogen: $P = 0.14$, $F_{1,30} = 2.36$

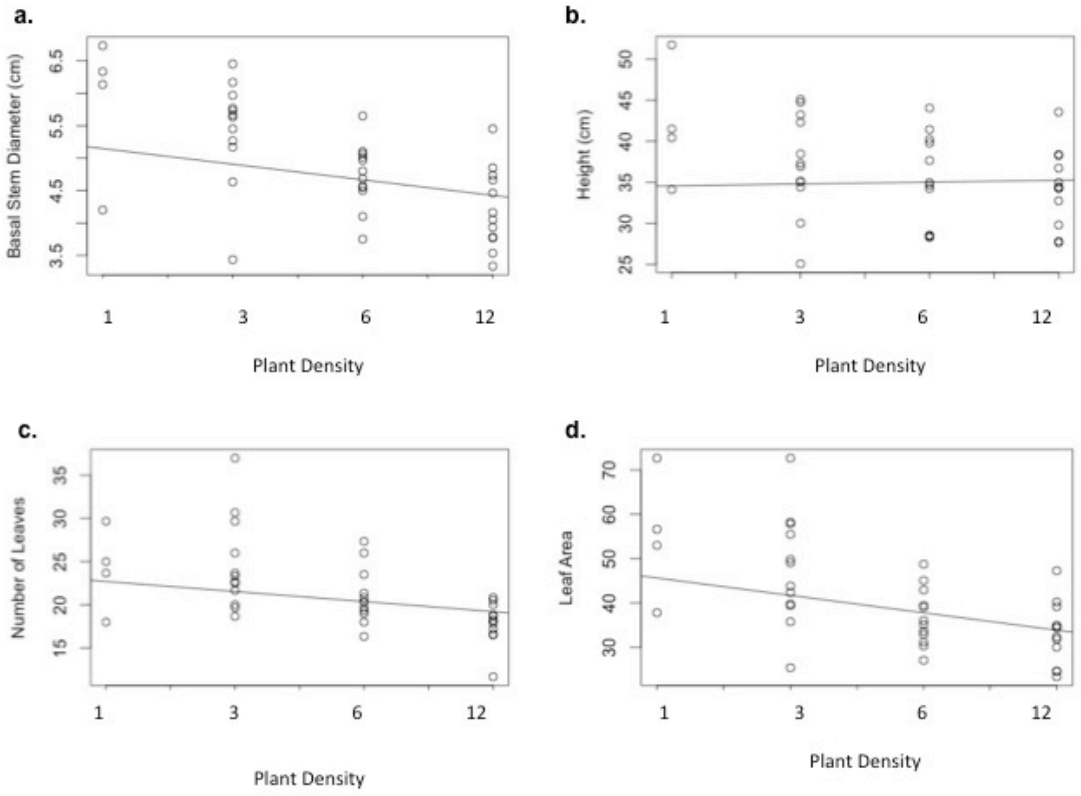


Figure 3. Intraspecific Competition Effects on Plant Size, Herbivory Intensity Study. Plant Density shown on x axis, leaf trait values on y axes. **(a)** Basal Stem Diameter (cm): β = -0.24, SE = 0.12, $P = 0.05$, $F_{1, 188} = 4.70$ **(b)** Height (cm): β = 0.23, SE = 0.92, $P = 0.80$, $F_{1, 38} = 0.06$ **(c)** Number of Leaves (per plant): β = -1.17, SE = 0.56, $P = 0.04$, $F_{1, 188} = 4.32$ and **(d)** Leaf Area (cm^2): β = -3.92, SE = 1.43, $P = 0.006$, $F_{1, 188} = 7.50$

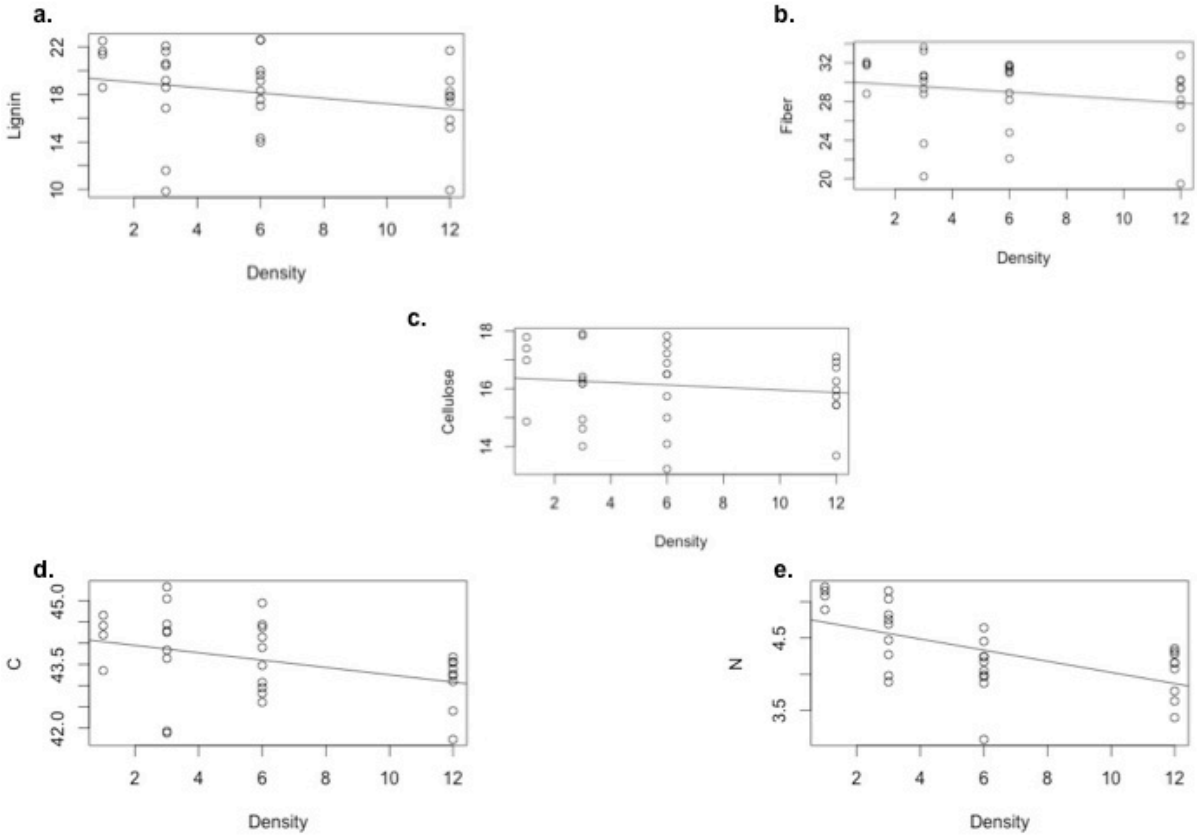


Figure 4. Intraspecific Competition Effects on Plant Leaf Traits, Herbivory Intensity Study. Plant density (x axis) plotted against leaf traits (y axes). **(a)** Lignin: β = -0.22, SE = 0.13, $P = 0.09$, $F_{1, 84} = 2.99$ **(b)** Fiber: β = -0.19, SE = 0.16, $P = 0.23$, $F_{1, 84} = 1.46$ **(c)** Cellulose: β = -0.04, SE = 0.06, $P = 0.47$, $F_{1,84} = 0.52$ **(d)** C: β = -0.09, SE = 0.03, $P = 0.01$, $F_{1, 84} = 6.31$ and **(e)** N: β = -0.08, 0.02, $P < 0.001$, $F_{1, 84} = 17.93$

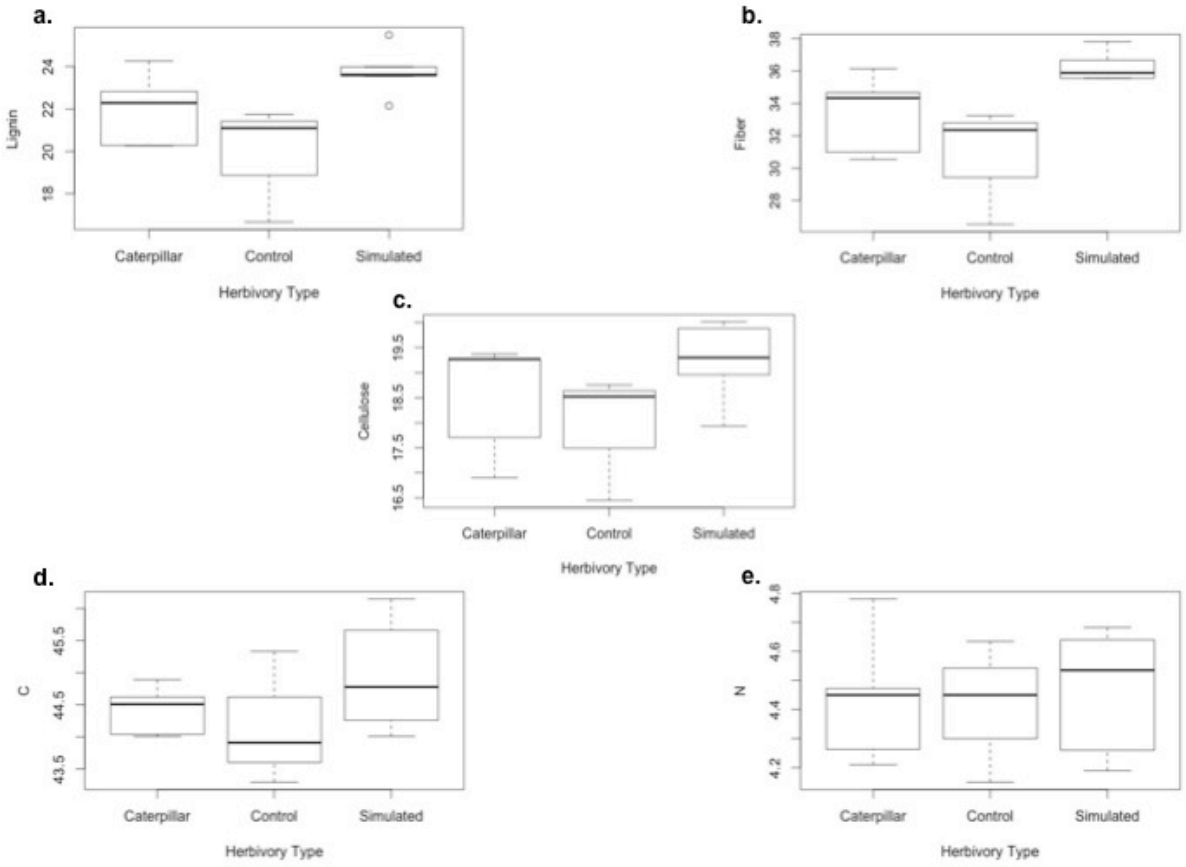


Figure 5. Herbivory Type Effect on Leaf Traits. These graphs show the effect of herbivory type (x axis) on leaf traits (Lignin, Fiber, Cellulose, C, and N).
(a) Lignin: $P = 0.002$, $F_{2,30} = 7.323$ **(b)** Fiber: $P = 0.001$, $F_{2,30} = 8.313$ **(c)** Cellulose: $P = 0.13$, $F_{2,30} = 2.20$ **(d)** C: $P = 0.02$, $F_{2,30} = 4.46$ **(e)** N: $P = 0.60$, $F_{2,30} = 0.54$

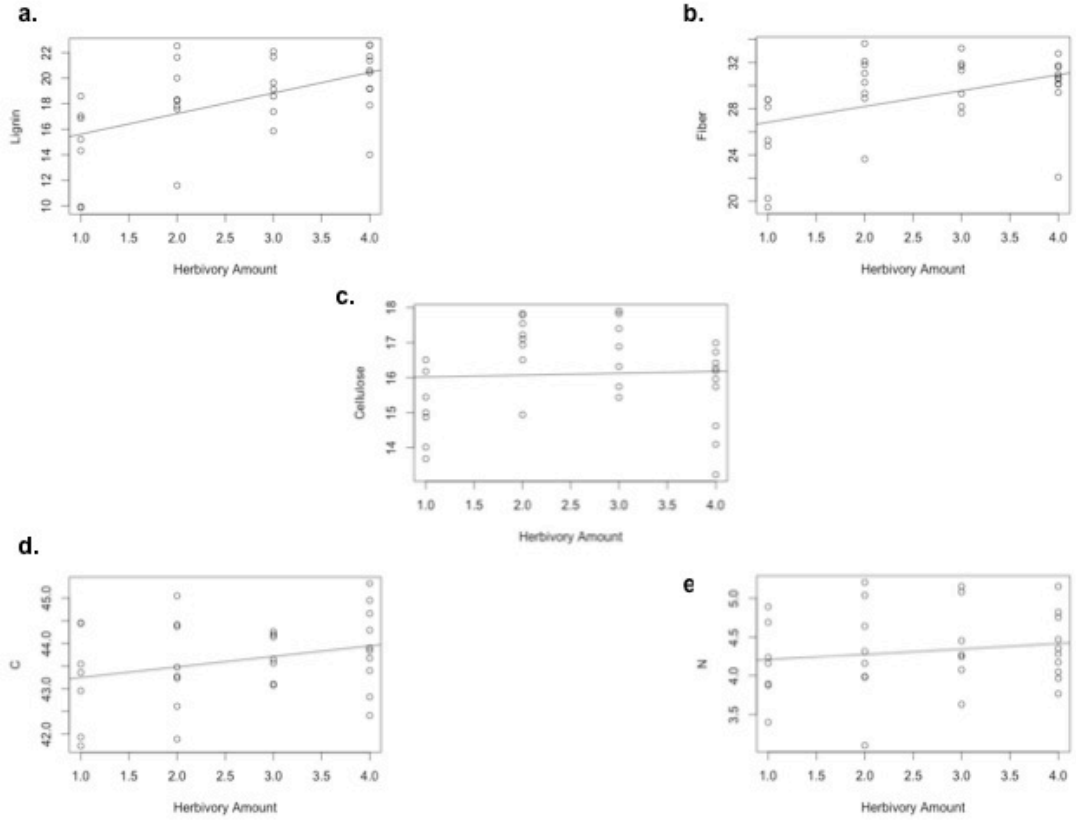


Figure 6. Herbivory Intensity Effects on Leaf Traits. These plots show herbivory amount (x axis) and leaf traits (y axis). Herbivory intensity corresponds to increasing damage (1 = no herbivory, 4 = high herbivory) **(a)** Lignin: β = 1.57, SE = 0.44, $P < 0.001$, $F_{1, 84} = 12.48$ **(b)** Fiber: β = 1.40, SE = 0.54, $P = 0.01$, $F_{1, 84} = 6.67$ **(c)** Cellulose: β = 0.07, SE = 0.22, $P = 0.74$, $F_{1,84} = 0.11$ **(d)** C: β = 0.26, SE = 0.12, $P = 0.04$, $F_{1, 84} = 4.48$ and **(e)** N: β = 0.07, SE = 0.07, $P = 0.32$, $F_{1, 84} = 1.01$

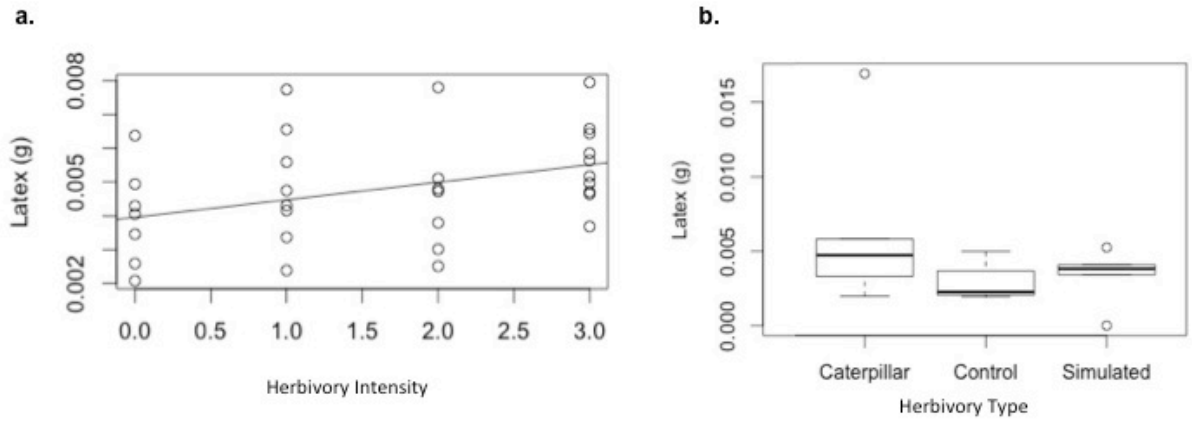
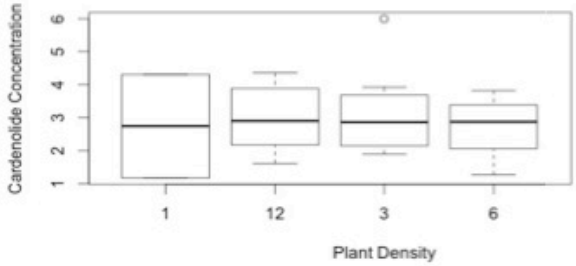


Figure 7. Herbivory Type and Amount Effects on Latex Exudation. (a) Herbivory Amount (x axis) effect on latex exudation (y axis) in *Asclepias syriaca.* Numeric values on the x axis correspond to increasing herbivory amount (0= no herbivory, 1= low herbivory, 2= medium herbivory, 3= high herbivory) β = 0.001, SE = 0.0002, $P < 0.001$, $F_{1,188} = 20.53$ **(b)** Herbivory Type (x axis) effect on latex exudation (y axis) in *Asclepias syriaca. P* = 0.298 $F_{2,54} = 1.24$

a.



b.

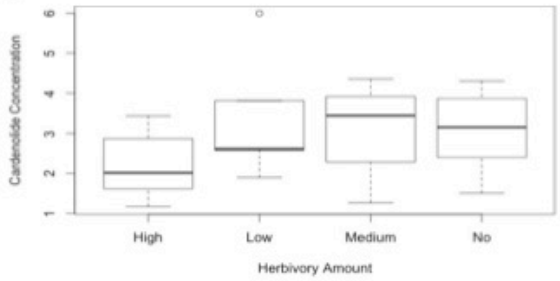


Figure 8. Cardenolide Responses in Herbivory Intensity Study. Figures a, b, and c show the cardenolide concentration (ug mg^{-1}) in samples based on **(a)** Plant Density $P = 0.759$, $F_{3,38} = 0.467$ **(b)** Herbivory Amount $P = 0.595$, $F_{3,38} = 0.64$

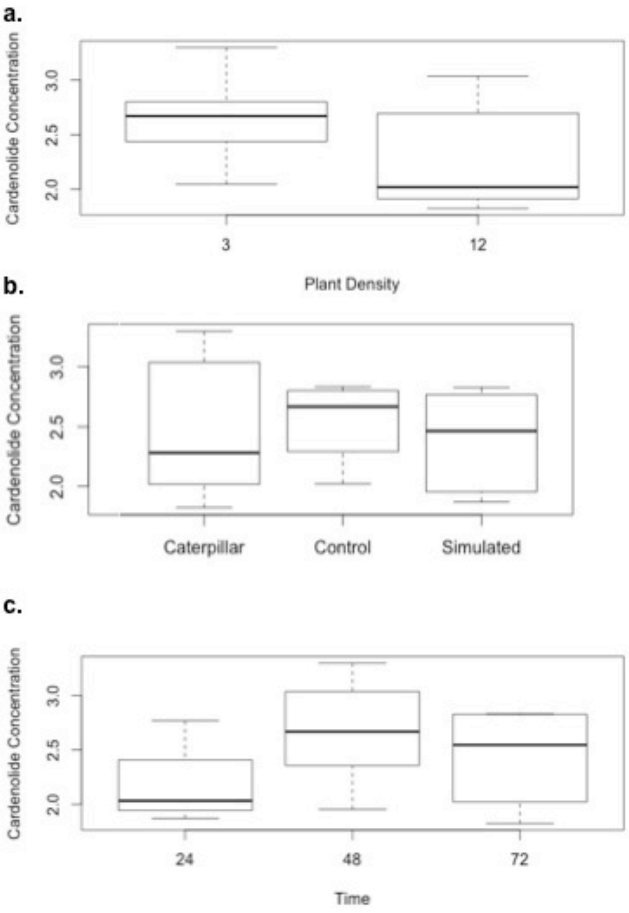


Figure 9. Cardenolide Responses in Herbivory Type Study. Figures a, b, and c show the cardenolide concentration (ug mg^{-1}) in samples based on **(a)** Plant Density $P = 0.179$, $F_{1,54} = 1.852$ **(b)** Herbivory Type $P = 0.847$, $F_{2,54} = 0.166$ and **(c)** Sampling Period $P = 0.341$, $F_{2,54} = 1.15$

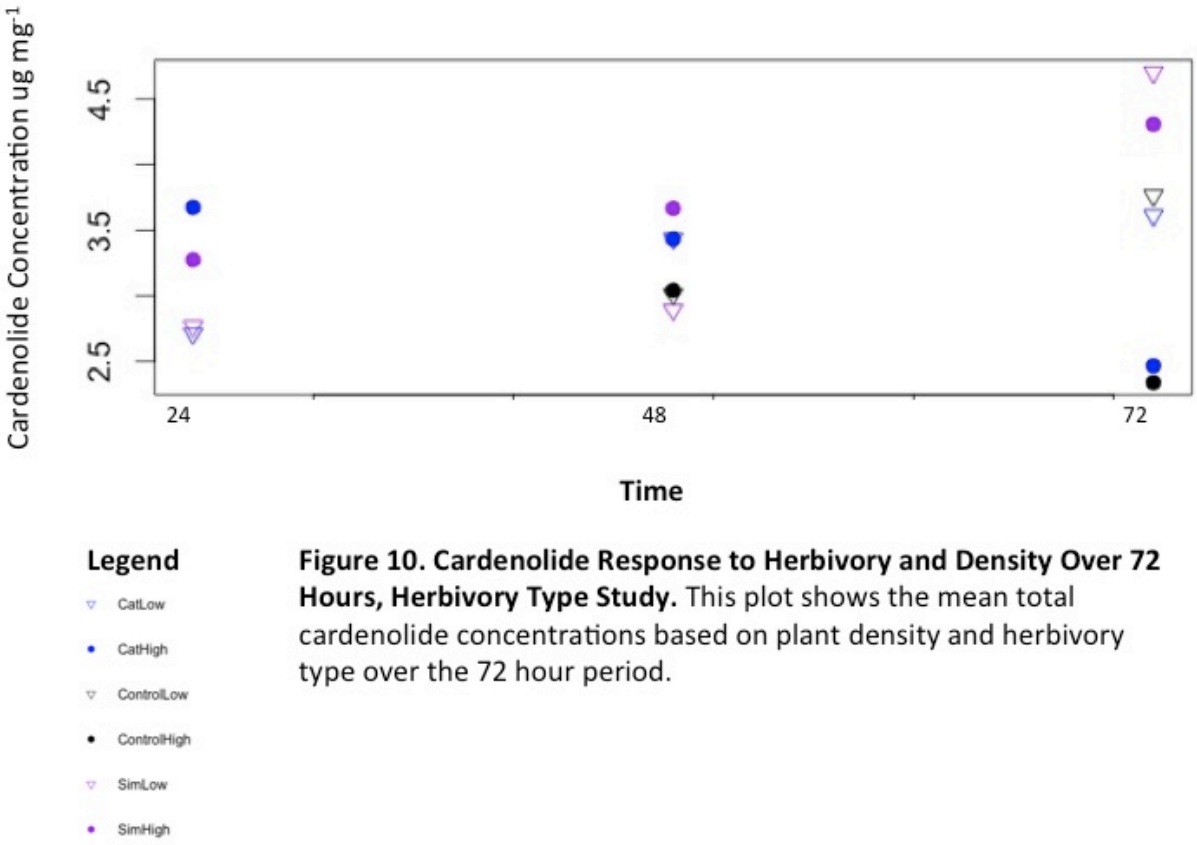


Figure 10. Cardenolide Response to Herbivory and Density Over 72 Hours, Herbivory Type Study. This plot shows the mean total cardenolide concentrations based on plant density and herbivory type over the 72 hour period.

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